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CLINICAL AND GENETIC STRATIFICATION OF CHILDHOOD PSORIASIS

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Clinical and genetic stratification of childhood psoriasis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Tro på dem som söker sanningen. Tvivla på dem som finner den.”

André Gide

ABSTRACT

Psoriasis is a clinically heterogeneous and common disease affecting about 2-3% of the population in Sweden. Twin and family studies have shown a strong and complex genetic background in psoriasis. The strongest known linkage association is for *HLA-C*06* on chromosome 6p21.3. During the last decade several genome-wide association studies (GWAS) have independently replicated strong association with *HLA-C*06* and found numerous other loci at which common single-nucleotide polymorphisms (SNPs) modestly influence the risk of developing psoriasis. Environmental factors such as infections, stressful life events and medication also contribute to disease development. Although psoriasis is such a highly heterogeneous disease most psoriasis susceptibility genes have been identified in cohorts of mixed clinical phenotypes and exploration of genes in clinical subtypes is scarce.

Age of onset is one factor that can be used for stratification. Onset of psoriasis can occur at any age in life but is most common in young adults and onset in childhood and adolescence is not uncommon. Studies stratifying for age of onset of psoriasis have in principle been limited to comparing onset before or after 40 years of age as defined by Henseler and Christophers in 1985. However, 75% of patients develop disease before 40 years of age, thus stratification for early onset of psoriasis using this definition comprises the majority of patients.

The overall aim of this thesis was to characterize the clinical phenotype and genetic background in psoriasis patients with disease onset in childhood and adolescence. Our hypothesis was that different genetic background could influence age of onset, clinical presentation and prognosis.

Within this PhD project patients with onset of psoriasis in childhood and adolescence have been included for careful clinical and genetic characterization. For stratification for age of onset patients were divided into four groups (0-9, 10-20, 21-40, > 40 years). This approach revealed different genetic variations even within the group of patients with onset before 20 years of age (0-9 vs. 10-20 years at onset) (*paper I, II and IV*). In *paper II* genetic association with *IL22* in early onset psoriasis was linked to functional alterations in IL-22 responses in T-cells. In *paper III* we describe clinical phenotypes at onset (< 12 months after onset) in children < 16 years. Pre-pubertal children more often had genital lesions, especially boys, whereas guttate phenotype and facial lesions associated with *HLA-C*06* which was more common in children with onset at puberty. Diagnosing psoriasis in potential children for inclusion was not always easy and eczema was the most common differential diagnosis.

In conclusion the data presented in this thesis stresses the importance of careful phenotypic characterization and stratification for age of onset in genetic studies in psoriasis.

Differences in genetic background could have an impact on the development of co-morbidities, treatment response and prognosis. Thus, exploration of the genetic background in stratified materials may help in determining prognostic genotype and tailored biologic treatment for patients in the future.

LIST OF PUBLICATIONS

- I. **Genetic association with ERAP1 in psoriasis is confined to disease onset after puberty and not dependent on HLA-C*06**
Josefin Lysell, Leonid Padyukov, Ingrid Kockum, Pernilla Nikamo, Mona Ståhle
Journal of Investigative Dermatology 2013 Feb; 133(2): 411-7
- II. **Genetic variants of the IL22 promoter associate to onset of psoriasis before puberty and increased IL-22 production in T cells**
Pernilla Nikamo, Stanley Cheuk, Josefin Lysell, Charlotta Enerbäck, Kerstin Bergh, Ning Xu Landén, Liv Eidsmo, Mona Ståhle
Journal of Investigative Dermatology 2014 Jun; 134(6): 1535-41
- III. **Clinical characterization at onset of childhood psoriasis – a cross sectional study in Sweden**
Josefin Lysell, Mesfin Tessma, Pernilla Nikamo, Carl-Fredrik Wahlgren, Mona Ståhle
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- IV. **Human leukocyte antigens in psoriasis patients stratified for age of disease onset**
Josefin Lysell, Henrik Källberg, Leonid Padyukov, Mona Ståhle, Pernilla Nikamo
Manuscript

PUBLICATIONS NOT INCLUDED IN THIS THESIS

- I. **Association with genetic variants in the IL-23 and NF- κ B pathways discriminates between mild and severe psoriasis skin disease**
Pernilla Nikamo, Josefin Lysell, Mona Ståhle
J Invest Dermatol. 2015 Mar 19. doi: 10.1038/jid.2015.103.
- II. **Lack of association between filaggrin gene mutations and onset of psoriasis in childhood**
M.C.G Winge, J.Suneson, J.Lysell, P.Nikamo, A.Liedén, M.Nordenskjöld, C.F. Wahlgren, M.Bradley, M.Ståhle.
J Eur Acad Dermatol Venereol. 2013 Jan;27(1):e124-7.
- III. **Antiviral therapy in children with Hydroa vacciniforme**
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Acta Dermato-Venereologica 2009 Jul;89:393-7.

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LIST OF ABBREVIATIONS

AD	Atopic dermatitis
AhR	Aryl hydrocarbon receptor
AS	Ankylosing spondylitis
BSA	Body surface area
CDLQI	Children's dermatology life quality index
CNV	Copy number variation
DLQI	Dermatology life quality index
DNA	Deoxyribonucleic acid
EDC	Epidermal differentiation complex
ER	Endoplasmic reticulum
ERAP1	Endoplasmic reticulum aminopeptidase 1
GWAS	Genome wide association study
HLA	Human leukocyte antigen
HWE	Hardy-Weinberg equilibrium
IBD	Inflammatory bowel disease
IL	Interleukin
INDEL	Insert/deletion
Kbp	Kilo base pair
LD	Linkage disequilibrium
MAF	Minor allele frequency
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK cell	Natural killer cell
PASI	Psoriasis area and severity index
pDC	Plasmacytoid dendritic cell
PGA	Physicians global assessment
PRP	Pityriasis rubra pilaris
PsA	Psoriatic arthritis
RF	Rheumatoid factor
SNP	Single nucleotide polymorphism
SPC	Stockholm psoriasis cohort
tTG	tissue Transglutaminase
TNF	Tumor necrosis factor
Tag SNP	Tagging single nucleotide polymorphism

1 BACKGROUND

1.1 PSORIASIS

1.1.1 Prevalence

Psoriasis is a chronic inflammatory skin disease, which affects approximately 2-3 % of the worldwide population [1-3]. The prevalence differs among populations, peaking in northern Europe and being less common in South America and Asia [4, 5]. Disease onset can occur at any age but onset in early adulthood is common and onset in adolescence and childhood is not uncommon. In approximately 30% of psoriasis patients disease appears before the age of 25 years [3, 6-8]. Onset before puberty is more unusual and psoriasis is estimated to occur in only 10% of patients before the age of 10 and in 2% before the age of 2 years, however these numbers are based on small and few studies [6, 9]. Health insurance data from Germany have shown an overall prevalence of 0.4% in individuals ≤ 18 years of age, prevalence ranged from 0.1% at the age of 1 year to 0.8% at the age of 18 years [10, 11]. In a study from Sweden > 8000 children aged 12-16 years were examined for dermatological disorders and prevalence of psoriasis was found to be 0.3% [12].

There is no gender difference in the prevalence of psoriasis. However, peaks of onset around hormonal transits like puberty and menopause implicate a hormonal effect in the development of psoriasis [13].

1.1.2 Type I and Type II psoriasis

In 1985 Henseler and Christophers presented a stratification of psoriasis patients into type I and type II psoriasis. This classification was in analogy to the classification of heritable type I diabetes and more sporadic type II diabetes. Type I patients had onset of disease before 40 years, more often carried human leukocyte antigen *HLA-C*06* and had a positive family history of psoriasis whereas type II patients included patients with onset after 40 years, less often carried *HLA-C*06* and lacked family history. However, 75% of patients develop disease before 40 years of age, thus stratification for early onset of psoriasis using this definition comprises the majority of patients [14].

1.1.3 Clinical features and quality of life

Psoriasis is clinically a highly heterogeneous disease with several phenotypes including plaque psoriasis, guttate psoriasis, pustular psoriasis, inverse psoriasis, erythrodermic psoriasis and palmo-plantar psoriasis. (*Figure 1*) Plaque type psoriasis is the most common phenotype in both children and adults and is characterized by well-demarcated rubrosquamous plaques. Erythrodermic psoriasis, pustular and palmo-plantar psoriasis are more uncommon phenotypes in children [7, 15, 16]. Guttate phenotype is most common in adolescent patients and has a more acute onset with widespread, smaller lesions developing over a few weeks, usually in conjunction with a streptococcal infection. Severity of disease

differs substantially over time and between patients. Also patients can switch from one phenotype to another.

Severity in psoriasis can be measured in different validated approaches, PASI (Psoriasis Area and Severity Index), PGA (Physicians Global Assessment) and BSA (Body Surface Area) [17]. PASI is based on the area involvement and rate of desquamation, induration and erythema of lesions and is the most frequently used assessment tool [18]. However, PASI does not take psychological aspects of localization of lesions into account. Small areas of psoriatic lesions localized in the face, which is more common in childhood psoriasis, or the dorsal of the hands can have a much larger social impact than lesions on the trunk that is covered with clothes.

Psoriasis is today considered a systemic disease and several co-morbidities are known. Patients with psoriasis have a higher prevalence of nail dystrophy, psoriatic arthritis (PsA), depression, Crohn's disease, uveitis, metabolic syndrome (overweight/obesity, hypertension, insulin resistance \pm glucose intolerance and dyslipidemia) and cardiovascular events compared to controls [19, 20]. In children and adolescents with psoriasis a higher prevalence of metabolic syndrome has been shown [10, 11, 21-23]. However, studies in the paediatric population show conflicting results regarding metabolic co-morbidities and further analyses are needed [24]. Early onset of psoriasis in childhood has not been shown to infer additional risk for cardiovascular and metabolic co-morbidities during adulthood [25].

Psoriasis is a chronic disease and impact on quality of life is significant and psychological co-morbidities like depression and anxiety are associated with psoriasis [10, 26, 27]. Onset of psoriasis early in life has been shown to have an age dependant impact on quality of life for patients and their family and to have a higher risk of psychological co-morbidities like anxiety and depression [28-30]. Quality of life is measured in a standardized and validated form for adults and children, dermatology life quality index (DLQI) vs. Children's dermatology life quality index (CDLQI).

Although treatment options and knowledge about underlying pathogenesis in psoriasis have increased immensely during the past decades, no cure for psoriasis exists and biomarkers for disease and for the risk of developing co-morbidities are lacking.



Figure 1. Plaque phenotype, guttate phenotype and pustular phenotype. All photos printed with permission from patients and parents. Photos taken by Medicinsk bild Karolinska University Hospital.

1.1.4 Histopathology

Psoriasis is a clinical diagnosis and histopathology can only guide us in diagnosing psoriasis, the histological picture is often uncertain when the clinical picture is atypical. Proliferation rate of keratinocytes is substantially increased in psoriasis and histologically, psoriasis show an increased thickening of the epidermis (*acanthosis*) with elongated rete ridges and incomplete keratinocyte differentiation of the upper layers, leading to retention of nuclei in the stratum corneum (*parakeratosis*). More dermal blood vessels are formed and a massive immune cell infiltrate can be observed both in epidermis and dermis, containing T cells, dendritic cells and others. The presence of neutrophils in the epidermis and especially in the stratum corneum, forming microabscesses, is characteristic for psoriasis histopathology. (Figure 2)

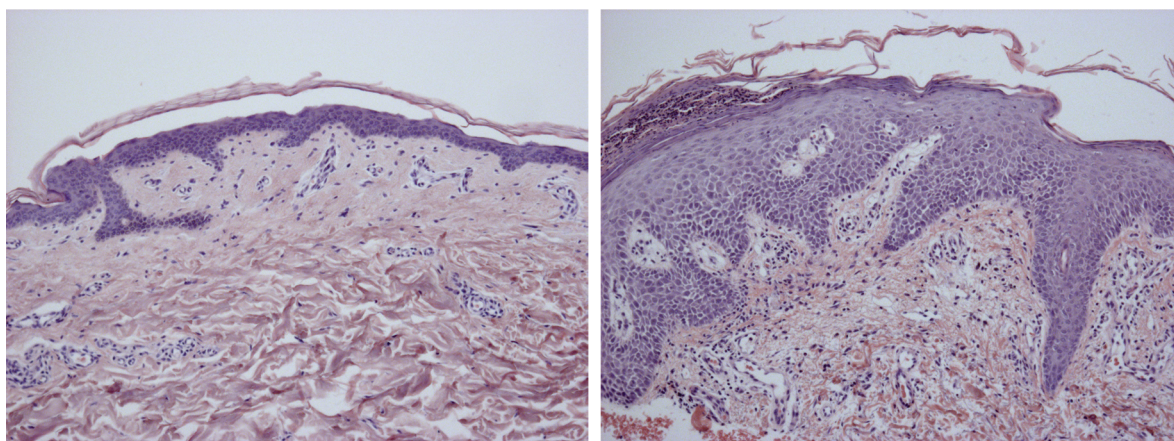


Figure 2. Histological section of healthy skin (left) and psoriasis lesional skin (right).

1.1.5 Psoriasis – a complex disease

The genetic background in psoriasis is strong and complex (*please see the monogenic vs. complex diseases section for details*). Monozygotic twins have a high concordance rate (up to 70 %) compared to dizygotic twins (up to 20 %) [31-33]. However, the lack of complete concordance in monozygotic twins indicates that additional environmental factors contribute to the development of psoriasis. Infections, especially streptococci, medication, stressful life events and smoking are some known environmental factors associated with psoriasis.

1.2 GENETICS

1.2.1 Deoxyribonucleic acid (DNA)

DNA molecules are large polymers with a backbone of sugar and phosphate and attached nitrogenous bases (A = Adenine, C = Cytosine, G = Guanine, and T = Thymine). In 1953 Watson and Crick published the first data on the structure of DNA showing that the DNA molecule forms a double helix with two antiparallel strands. [34] Stability between the two strands is maintained through hydrogen bonds between bases; A and T forming two hydrogen bonds and C and G forming three.

The genetic code corresponds to proteins and the fundamental unit of this code is termed a codon, which consists of three nucleotides that code for an amino acid, the building blocks of proteins. A gene is a sequence of nucleotides located in a particular position (locus) on a chromosome that encodes a functional product, e.g. a protein. In humans, the DNA helix is organized in 23 pairs of chromosomes. We have two copies of each 22 autosomes (all chromosomes except sex chromosomes), one inherited from our mother and one from our father. A person having two identical gene copies (alleles) in a region (locus) is said to be homozygous, whereas a person with two different alleles is said to be heterozygous. The human genome contains ~ 3 billion base pairs, approximately 1-2% codes for genes [35]. In 2001, the first analyses of the human genome sequence were published [35, 36].

Over the last decades the importance of non-coding sequences and regulatory elements like microRNA and long non-coding RNA in gene regulation has become established. Epigenetic modifications influenced by environmental factors also affect the expression of our genes. Whether such epigenetic modifications to some extent could even be heritable remains debatable but data supporting heritable changes exist [37].

1.2.2 Meiosis and recombination

Meiosis is the cell division leading to the formation of gametes. While the importance of mitosis (cell division leading to two exact copies of the cell) is to ensure the number of cells in the organism and keep the cells diploid the importance of meiosis is to make them haploid, which in humans involves reducing the chromosome count from 46 to 23. Meiosis involves separation of the chromosomes in each pair, but before that also an exchange of genetic material between homologous chromosomes in a pair can occur. (*Figure 3*) This process of genetic recombination helps preserve genetic variability within a species.

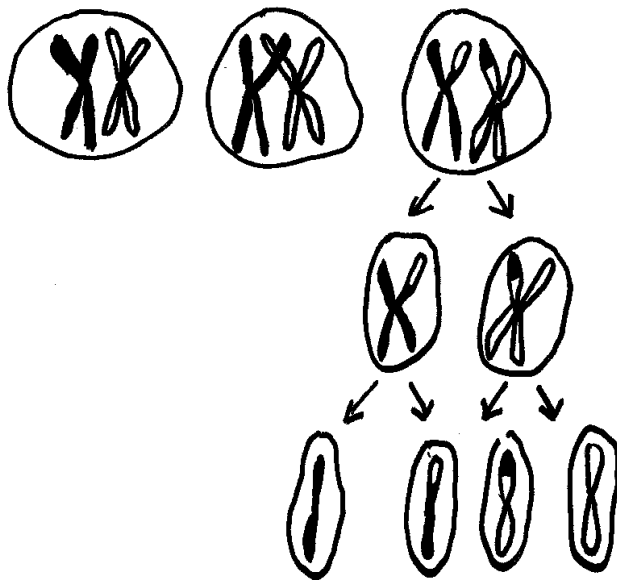


Figure 3. During meiosis recombination, or crossover, can occur between homologous chromosomes. This process enable exchange of genetic material producing gametes with unique mixture of the two chromosomes.

1.2.3 Monogenic diseases vs. complex diseases

According to Mendel's laws of inheritance, a phenotype (e.g. a disease) shows a dominant inheritance pattern if it manifests in a heterozygous individual (two different alleles at a given locus) and a recessive inheritance pattern if it only manifests in homozygous individuals (two identical alleles at a given locus). Most monogenic disorders (caused by a mutation in a single gene) follow these laws but multifactorial or complex diseases, such as psoriasis and eczema, do not follow any simple mode of inheritance. [38] These diseases have a clear heritable component but instead depend on a number of genes and environmental factors. In complex polygenic diseases each gene may have a very small effect on its own and gene-gene interactions (epistasis) and gene-environmental interactions may be involved.

1.2.4 Genetic variation

With almost 99.9% of the genomic composition being identical between humans, the remaining 0.1% still consists of millions of base pairs varying among different individuals [39].

A mutation is defined as any change in a DNA sequence away from normal. This implies there is a normal allele that is prevalent in the population and that the mutation changes this to a rare and abnormal variant. A point mutation has a deleterious effect on the individual and is responsible for an aberrant protein or no protein at all.

In contrast, a polymorphism is a DNA sequence variation that is common in the population. In this case no single allele is regarded as the standard sequence. Instead there are two or more equally acceptable alternatives. The most common allele is called major allele and the less common minor allele.

The arbitrary cut-off point between a mutation and a polymorphism is 1%. That is, to be classed as a polymorphism, the least common allele must have a frequency of 1% or more in the population. If the frequency is lower than this, the allele is regarded as a mutation.

A number of variations (polymorphisms) are commonly found in the genome. These include single nucleotide polymorphisms (SNPs) (GGGTT → GGCTT), insertions or deletions of single nucleotides or nucleotide-stretches of various length (INDELS) (GGGTT → GGT), length variations of tandem repeated sequences (micro and mini satellites) and copy number variations (CNVs).

SNPs are the most abundant form of genetic variation in the human genome, at least 3.1 million SNPs or ~1 every 1000 bases. There is a range of biological and phenotypic effects depending on variation. For example, the substitution of a nucleotide in a protein coding sequence may alter the amino acid sequence of the protein and changed nucleotide sequence in a regulatory region may change the expression of the protein. Polymorphic sequence variants usually do not cause overt debilitating diseases. However, genetic variation is an important basis for the phenotypic differences seen among individuals and is sometimes associated with development of diseases and can influence drug responses.

1.2.5 Linkage disequilibrium

During meiosis, crossing-over between loci on two homolog chromosomes occurs, i.e. recombination. Recombination is more likely to occur if two loci are distant and thus more unlikely the closer the loci are. Thus polymorphisms in close proximity tend to be inherited together. This correlation along a chromosome is known as linkage disequilibrium (LD). LD is usually measured pair-wise between SNPs, either by using r^2 or D' . Both measures range from 0 (equilibrium) to 1 (complete disequilibrium). The measure r^2 represents a statistical correlation between two SNP sites, whereas D' less than 1 indicates that recombination has occurred between the sites [40]. In addition to the inverse correlation to distance between markers LD is also variable in different genomic regions [41]. Knowing the LD structure in a genetic region of interest is essential for the construction of haplotypes (*please see next section*). Strong LD over a region is commonly referred to as a haplotype block [40]. LD is influenced by a number of factors including the rate of mutations, natural selection and random drift, the recombination fraction, non-random mating and migration.

1.2.6 Haplotypes

A haplotype is a group of genes within an organism that was inherited together from a single parent. This group of genes was inherited together because of genetic linkage. The set of genes residing on a specific chromosome or a specific part of a chromosome is called a haplotype. For example, for two SNPs with the alleles A/G and C/T there are four different possible haplotypes for that region: A-C, A-T, G-C and G-T. The frequencies of the haplotypes depend on the frequencies of each allele in the population. New haplotypes on a chromosome arise by the accumulation of additional mutations and recombination events. Polymorphisms that uniquely identify haplotypes are called tagging SNPs (tag SNPs). The

haplotype map of the human genome is available through the “HapMap project” database (www.hapmap.org). HapMap offers the possibility of using tag SNPs within a haplotype block hence, limiting the number of SNPs to be used in an association study. By genotyping a SNP in a LD block, sufficient genotype information is captured for all the correlating markers within this block. Since the combination of different genetic variants in a region might have a different impact on a phenotype, compared to their individual effects, the identification and study of haplotypes is therefore the aim in many genetic studies.

1.2.7 Association studies

In 1996 Lander postulated that common complex diseases are caused mainly by common variants with moderate effects that are quite frequent in the population [42]. This hypothesis is called the “common disease – common variant” and is still used today.

Genetic association studies are used to find candidate genes or genome regions that contribute to a specific disease by testing for a correlation between disease status and genetic variation. Association studies compare allele and genotype frequencies between cases with disease compared to controls. A significantly higher frequency of one or the other allele in cases relative to controls indicates an increased risk for disease in the presence of the specific allele [43]. Since association studies compare allele and genotype frequencies, it is important to ensure a common genetic background for cases and controls so that any observed differences are due to presence or absence of disease and not to population stratification.

Genome wide association studies (GWAS) interrogate the whole genome at once using SNPs and have become a popular method for the investigation of common disease associated variants. GWAS are designed to provide a survey of common variation [minor allele frequency (MAF > 0.05)], therefore examining only a portion of the genomic landscape of complex traits. Low-frequency (MAF 0.01-0.05) and rare (MAF < 0.01) variation has thus far been more challenging to access. The importance of rare variants in common diseases is today being recognized and GWAS studies using larger numbers of SNPs for better coverage and the development of high-throughput sequencing has increased our possibilities to find rare variants involved in disease development.

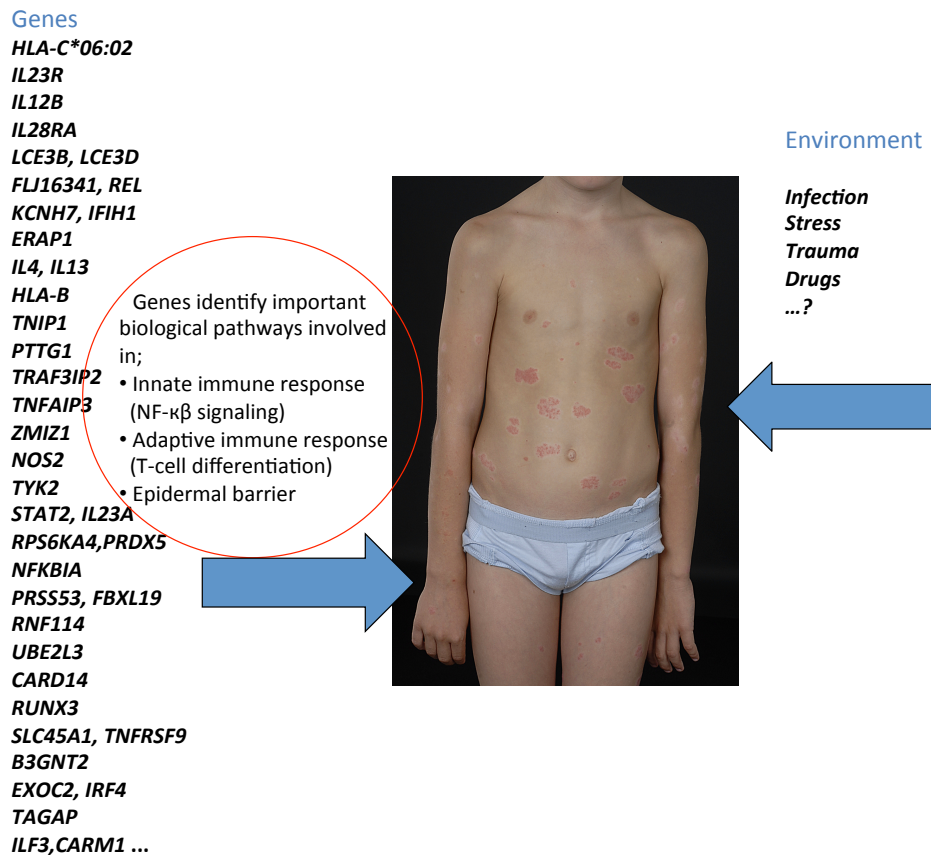
1.2.8 Hardy-Weinberg equilibrium (HWE)

The English mathematician G. H. Hardy and the German physician W. Weinberg postulated an important formula for genetics of populations in 1908. They concluded that the expected genotypes of a random mating population can be calculated using allele frequencies of that specific population [44]. Of the alleles A and a, a combination of these must exist at a specific loci in each individual. Thus the probability of finding either A or a, at a specific loci in an individual equals 1, $A + a = 1$. Hardy and Weinberg named the frequencies p and q, $p + q = 1$. Each individual carries two parental alleles at a given locus called the genotype of that individual. Thus an individual will carry the genotype AA, Aa or aa at a given loci, $p^2 + 2pq + q^2 = 1$. These two equations; $p + q = 1$ and $p^2 + 2pq + q^2 = 1$ forms Hardy Weinberg formula and applies to populations in genetic equilibrium. A test for HWE using chi-square

test or Fischer's exact test is primarily made as a quality control of genotype data in association studies.

1.2.9 Genetics in psoriasis

Psoriasis displays a complex genetic background with several genes involved in disease development. A total of 36 disease-associated loci have been identified and replicated in larger studies as contributing to psoriasis [45, 46].



Most genes associated with psoriasis are genes involved in the innate and adaptive immune system (*please see chapter "The pathogenesis of psoriasis"*). Genetic deletion of late cornified envelope (LCE) genes on chromosome 1q21 in the epidermal differentiation complex (EDC), involved in differentiation of keratinocytes, represents a skin specific susceptibility gene for psoriasis [47].

Most associated genes display a modest increase in OR except for the strongest associated gene in psoriasis *HLA-C*06* on chromosome 6. Strong linkage disequilibrium in the HLA region on chromosome 6 made identification of the true susceptibility gene challenging for researchers. However, in 2006 Nair et al. presented sequence and haplotype analysis data which strongly supports *HLA-C*06* to be the psoriasis susceptibility gene [48]. HLA-C is expressed by all nucleated cells and presents intracellular proteins (antigens) to CD8^{pos} T cells. Also keratinocytes express HLA-C and are thought to interact via this molecule with

natural killer (NK) cells [49]. (please see *HLA chapter below*) In Sweden 11-12% of the population carry the *HLA-C*06* antigen in comparison to ~ 40-60% of psoriasis patients [50-52].

Association with endoplasmic reticulum aminopeptidase 1 (*ERAP1*) in psoriasis was published in a GWAS in 2010 [53]. Association with *ERAP1* was confined to patients carrying *HLA-C*06* and a statistical interaction between these loci were found. ERAP1 is involved in trimming of peptides in the endoplasmic reticulum (ER) before transportation of peptides to the cell surface for presentation on HLA molecules. (Figure 4) ERAP1 has also been shown to be involved in shedding of pro-inflammatory cytokine receptors (tumor necrosis factor receptor 1 (TNFR1) [54], IL-1R2 [55] and IL-6R α [56], altogether suggestive of a potential involvement in the pathogenesis of psoriasis.

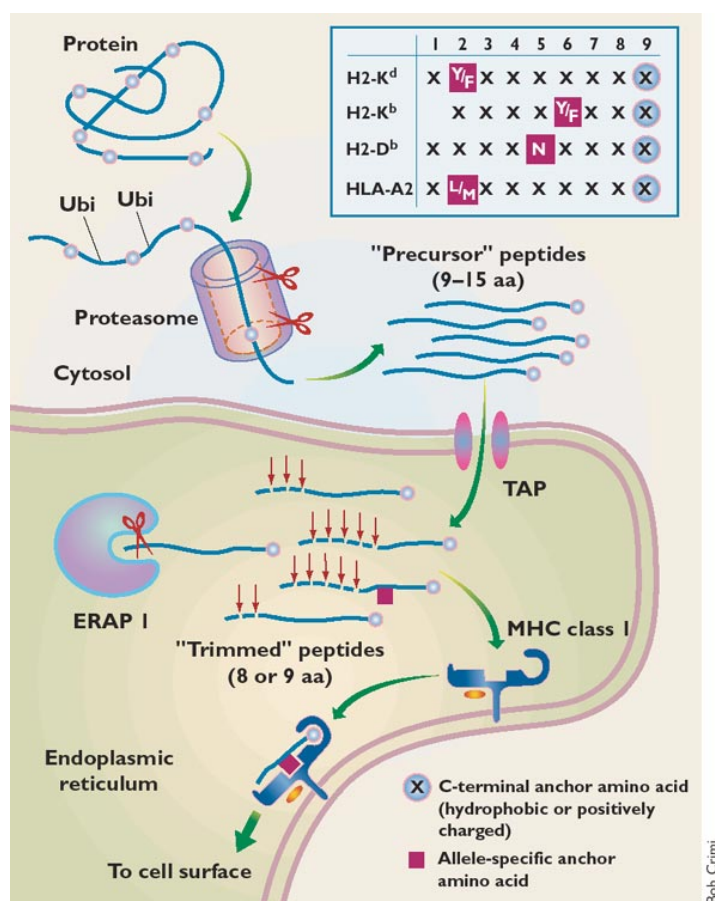


Figure 4. MHC class I processing pathway. Reprinted by permission from Macmillan Publishers Ltd: *Nature Immunol.* 3(12):1121-2, copyright (2002).

In some families mutations in *IL-36RN* have been reported to associate with severe pustular psoriasis [57, 58]. The anti-inflammatory effect of the IL-36 receptor antagonist is decreased by the mutation. Thus, even though psoriasis is generally considered as a complex disease, some phenotypes may appear as monogenic traits, highlighting the genetic heterogeneity underlying psoriasis.

The genes are the templates in our cells but regulation of expression of our genes exists at several different levels. Epigenetic changes and microRNAs have been shown to regulate gene activity in psoriasis [59-63]. Interestingly, a recent publication found immune cells and proteins in healthy human twins to vary because of non-heritable influences (vaccines, microbes etc.) with only minor influence from heritable factors [64].

1.2.10 Human leukocyte antigens

1.2.10.1 Function

The immune system must discriminate between self and non-self antigens and react to non-self but not to self-antigens. To be able to do this, all nucleated cells have surface antigens that are specific for that individual. In autoimmune diseases there is a break of tolerance towards self-antigens resulting in an incorrect immune response to self-antigens. The human leukocyte antigens (HLA) were early recognized for its extreme diversity and for its association with many autoimmune and infectious diseases. The genes encoding HLA are localized in a gene cluster on the short arm of chromosome 6 called the major histocompatibility complex (MHC). This region is very gene dense with an average gene density of 1 gene per 16 kbp. This should be compared to 1 gene per 60 kbp in the rest of the genome. Many genes located in the HLA region are involved in immunological functions but the region also contains other genes not involved in the immune response.

The HLA region is divided into class I, class II and class III regions. The class I region, at the telomeric end of the HLA region, contains genes encoding the classical antigens HLA-A, B and C. The class II region, in the centromeric part of the HLA region, contains HLA-DR, DQ, DP. The class III region is located between HLA class I and HLA class II, this region contains genes in the complement system, C2 and C4 and also the tumour necrosis factor (TNF), which is a potent cytokine. (*Figure 5*)

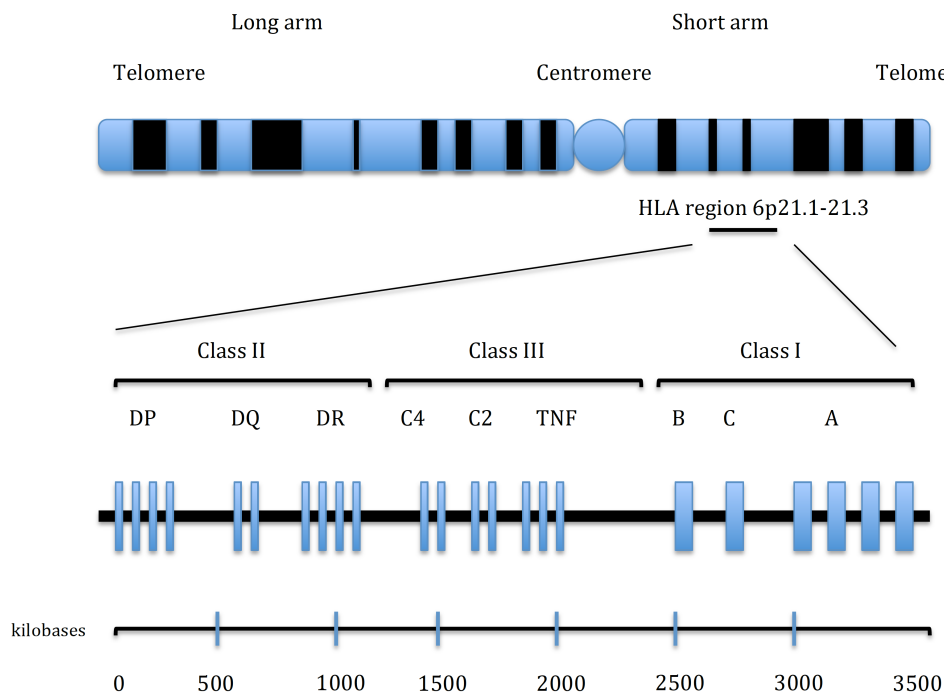


Figure 5. The HLA Complex on the short arm of chromosome 6

1.2.10.2 Structure

Class I and class II molecules are transmembrane glycoproteins acting as receptors in the immune system. Class I molecules are composed of two polypeptides, a longer α -chain and forming into 3 domains and a shorter β_2 -chain. The two outer domains of the heavier α -chain form the antigen-binding pocket. (Figure 6) Class I molecules are expressed on the surface of all nucleated cells and present intracellular peptides both from self proteins and viruses processed in the cell. The T-cell receptor on CD8+ cells (mostly cytotoxic cells) recognises antigens presented by class I molecules. These peptides are 7-10 amino acids in length and are processed in the ER to the right length. From the ER the HLA-class I molecule and antigen complex is transported to the cell surface for presentation. The T-cell receptor (TcR) recognises the conjunction of HLA-molecule and presented peptide.

HLA class-II is composed of two equally long polypeptides, an α -chain and a β -chain. (Figure 6) The outer domain of the β -chain is highly polymorphic and it is mainly in this part the antigen binds specifically. [65, 66] HLA-class II molecules are expressed on professional antigen-presenting cells (B-cells, macrophages, dendritic cells and monocytes) and tissue specific cells that also present antigens. HLA-class II molecules present exogenous peptides from extracellular proteins that have been endocytosed and processed in the cell.

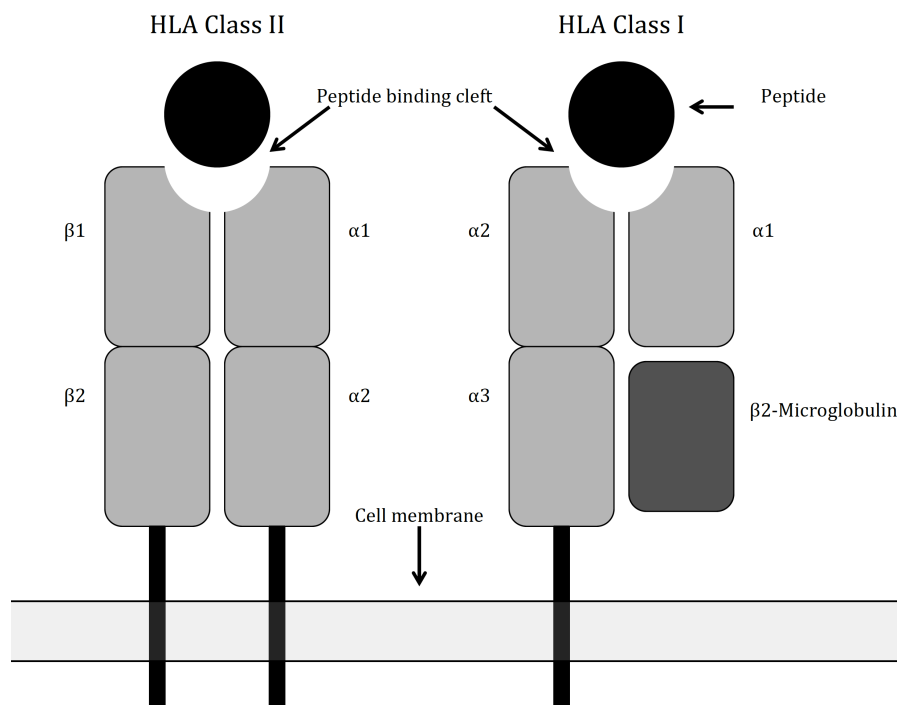


Figure 6. The HLA class I and class II molecules

1.2.10.3 Polymorphism and linkage disequilibrium in the HLA region

The HLA class I and II products are among the most polymorphic proteins known in humans. The number of known alleles for the HLA-C locus is today > 2500 and ~ 150 for *HLA-C*06*. These polymorphisms reflect differences in the specificity of the antigen-binding site; the variation in the molecule affects its affinity to different antigenic peptides. This diversity is important to mankind with high number of alleles in the population increasing the defence against pathogens in the population as a whole.

1.3 THE PATHOGENESIS OF PSORIASIS

The immune response can be divided into the innate and the adaptive immune system, both involved in psoriasis pathogenesis. The innate immune system, also known as the non-specific immune system, is the first line of defence against pathogens and includes neutrophils, cytokines, chemokines, toll-like receptors and antimicrobial peptides. The adaptive immune system, also known as the acquired immune system or the specific immune system creates immunological memory (memory B and T cells) after an initial response to a specific pathogen (presented by antigen presenting cells) and subsequent encounters with that pathogen leads to an enhanced response. The adaptive immune system includes both humoral immunity components (such as antibodies, complement proteins and antimicrobial peptides) and cell-mediated immunity components.

The pathogenesis of psoriasis is not fully understood, but extensive research has led to a model where interaction between keratinocytes and immune cells establish a vicious cycle of chronic inflammation. This process has been described as the IL-23/T_H17/IL-22 axis of psoriasis [3, 67]. (Figure 7)

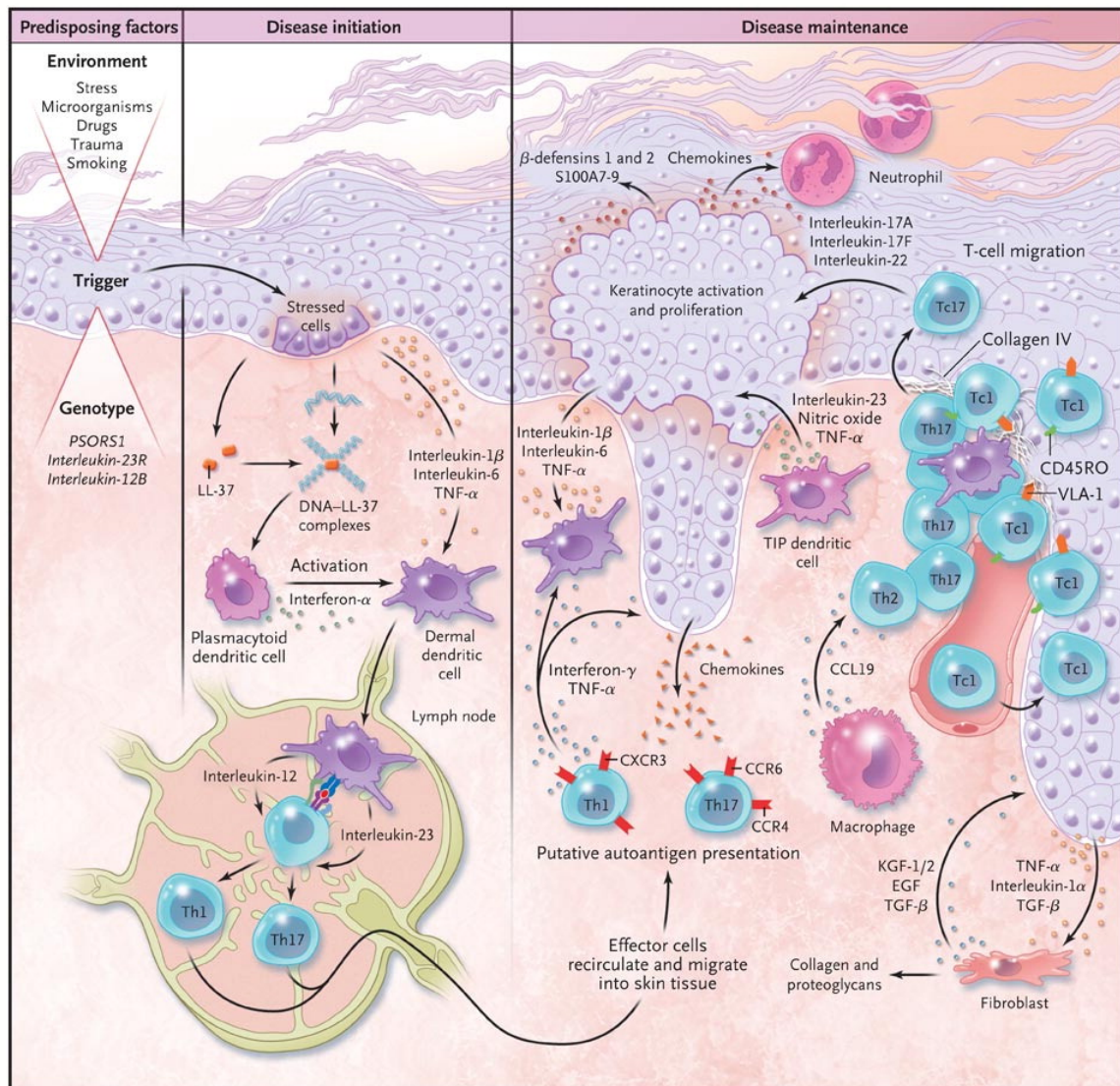


Figure 7. Model of the pathogenesis of psoriasis by the IL-23/T_H17/IL-22 axis. Reproduced with permission from (Nestle et al., 2009b). Copyright Massachusetts Medical Society.

I will here try to summarize this proposed model of psoriasis pathogenesis;

The innate immune system is activated in the skin in genetically predisposed individuals exposed to environmental triggers like infection or trauma. The initial spark initiating the inflammatory cascade in genetically predisposed individuals has not been known. However, data supports LL-37 and self-DNA complexes to be a potential explanation of the mechanism through which host DNA is turned into a pro-inflammatory stimulus that breaks immunologic tolerance in psoriasis [68-70]. The hypothesis is that complexes of the antimicrobial peptide LL-37 cathelicidin and self-DNA released from necrotic cells in the stressed skin activate plasmacytoid dendritic cells (pDCs) and starts of the inflammatory cascade in psoriasis [71]. Psoriatic keratinocytes are a rich source of antimicrobial peptides, including LL-37, β-defensins, and S100A7 (psoriasin). The initial phase involving the innate immune system includes neutrophils, secretion of cytokines like IL-1β, IL-6, TNF-α, interferon-α and

activation of pDCs. Subsequently myeloid dendritic cells (mDCs) are activated in the local inflammatory milieu in the skin and migrate to the draining lymph nodes and induce the differentiation of naive T cells into effector cells such as type 17 helper T cells (T_H17) or type 17 cytotoxic T cells (T_C17) and type 1 helper T cells (T_H1), although it remains debatable what antigen might be presented during this interaction. LL-37-specific T cells have been found in psoriasis patients and LL-37 has been proposed to be the autoantigen involved in the activation of the adaptive immune system in addition to its activation of the innate immune system [72]. Activated mDCs, but also keratinocytes produce high levels of IL-23, which favours the polarization and maintenance of T_H17 cells and is found in increased concentrations in psoriasis lesions [73, 74]. T_H17 and T_H22 cells recirculate and migrate into the skin from the lymph nodes and contribute to an inflammatory reaction. T_H17 and T_H22 cells produce a series of pro-inflammatory cytokines, most prominent IL-17 and IL-22, which have major effects on keratinocytes. The activation of keratinocytes by IL-17 and IL-22 leads to hyperproliferation [75, 76] and to the production of chemokines [77, 78] that in turn attract more immune cells into the skin. Thus, a vicious cycle of continuous inflammation is maintained.

1.3.1.1 IL-22

The skin is covered by microorganisms [79], and is constantly surveyed by memory T cells [80]. Circulating T cells are pulled into spots of even microscopic inflammation [81], creating constant interactions between the skin immune cells and microbial products [82]. IL-22 is proposed to act as a gatekeeper maintaining immune homeostasis at barrier surfaces like the skin, gut or respiratory mucosa through induction of innate antimicrobial genes [77, 78, 83, 84]. IL-22 induces the production of a series of granulocyte-attracting chemokines and antimicrobial peptides. Moreover, IL-22 inhibits differentiation and cornification of keratinocytes in the epidermis [85].

IL-22 is highly expressed by immune cells such as T cells in several chronic inflammatory conditions, including psoriasis [75, 86]. IL-22 signals via a receptor consisting of IL-22R and IL-10R2 subunits [87]. In contrast to IL-22-producing cells, the IL-22 receptor is exclusively expressed on non-immune cells such as epithelial cells. Thus, IL-22 is uniquely positioned in the communication between the immune system and the epithelium, reflecting its key role in host defence and antimicrobial protection [84].

Environmental stimuli are known to contribute to psoriasis pathogenesis and that of other autoimmune diseases, but the mechanisms are largely unknown. The aryl hydrocarbon receptor (AhR) is a transcription factor that senses environmental stimuli. AhR is a ubiquitous transcription factor present in the cytoplasm. Upon ligand binding, AhR translocates into the nucleus where it regulates the expression of a variety of genes including *IL22*. AhR binds to and is activated by a range of structurally divergent chemicals including natural dietary, endogenous ligands and synthetic environmental agents among which dioxin (2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD)) is the most extensively studied pure agonist [88]. Interestingly AhR is implicated as a master regulator of IL-22 production [89-91].

2 AIMS

The overall aim of this thesis was to clinically characterize children with early onset of psoriasis and to explore the genetic background in childhood and adolescent onset of psoriasis.

In particular the objectives of this work are:

- To explore age dependent differences in association with two previously associated genes in psoriasis, *HLA-C*06* and *ERAP1*.
- To study genetic association with *IL22* in our psoriasis cohort including stratification for age of onset and functional effects of carrying these genetic variants.
- To clinically characterize children with early onset of psoriasis (< 16 years) at onset (within 12 months after onset) and to explore if genetic differences between onset before or after puberty translate into clinical differences.
- To investigate age dependent differences in association with human leukocyte antigens in psoriasis.

3 MATERIALS AND METHODS

3.1 SUBJECTS

The Stockholm Psoriasis Cohort (SPC) was initiated in 2000 at department of dermatology at Karolinska University Hospital. The cohort comprises more than 700 individuals included within 12 months of onset of psoriasis. From the beginning only patients older than 15 years of age were included and clinical characterization of the adult cohort has been published. [92] After receiving ethical permission, children ≤ 15 years with onset of psoriasis within 12 months have been included. Recruitment mainly occurred between 2007-2014 and is still ongoing. Children have been included through consecutive recruitment from new referrals and follow-up visits at the department of dermatology at Karolinska University Hospital and from the psoriasis patient association care units in Stockholm. Inclusion included clinical examination, physician-led interview and blood sampling (for more details please see *paper III*). Several children examined for inclusion failed to fulfil the inclusion criteria of onset < 12 months because of delayed diagnosis and numbers to reach power in genetic studies were difficult to obtain. For genetic studies the cohort was supplemented with patients with onset of psoriasis before 15 years of age and > 12 months of disease duration. These patients had to have an unequivocal psoriasis lesion at inclusion and were not allowed to be older than 30 years at inclusion to minimize recall bias. Only scalp psoriasis was not regarded as sufficient for inclusion.

The SPC includes a cross-section of patients with disease severity ranging from mild to severe with a predominance of mild cases. [92] During the past decade several new systemic therapies for psoriasis were introduced and systemic treatment for psoriasis has become more common. Most patients with psoriasis cope with local treatment and systemic therapy is reserved to patients with severe psoriasis. Since 2007 psoriasis patients starting on systemic treatment in the dermatology department at Karolinska University Hospital are included in a cohort of severe psoriasis patients for clinical and genetic studies. These patients have also been included in the genetic studies within this thesis. To ensure that patients enrolled in more than one of the cohorts have only been used as one case in the studies controls for this have been made.

For replication of association findings in *paper II* a group of patients with onset of psoriasis between 0-9 years ($n = 98$) were provided from professor Charlotta Enerbäck's group at Linköping University.

All studies in this thesis were approved by the Regional Ethical Review Board in Stockholm, Sweden. Oral and written consent was obtained from all included patients and for children < 18 years consent was also obtained from parents. At least one of the parents was in command of the Swedish language.

3.2 CONTROLS

Controls used in the studies are; **I**, controls for the adult patients (> 16 years at onset) in the SPC cohort matched for residential area, age and sex (*paper I and II*) [92] **II**, controls from the Epidemiological Investigation of Rheumatoid arthritis study (EIRA study) [93] **III**, healthy blood donors at the Karolinska University Hospital.

3.3 METHODS

DNA was extracted from blood using Puregene blood core kit (Qiagen). DNA concentrations were determined by Nanodrop (Thermo Fisher Scientific Inc, Wilmington, DE, USA).

Most of the genotyping was performed with TaqMan® SNP Genotyping Assays (Applied Biosystems (ABI)). This method is based on allelic discrimination of the wild type allele and the variant allele by two minor groove binder (MGB) probes. Each of the two probes is labelled with a different fluorescent dye, FAM or VIC. Primers and probes were obtained from ABI. Allelic discrimination was performed with the ABI PRISM® 7900HT Sequence Detection System (SDS) and the SDS 2.2.1 program (Applied Biosystems).

PCR with sequence-specific primers (PCR-SSP) was used for HLA typing. The primers are designed to have perfectly matched 3'-ends with a single allele or a group of alleles. During PCR amplification a perfectly matched primer pair results in amplification. With gel electrophoresis and documentation by photography of UV luminescent bands the presence or absence of DNA bands are visualized. HLA-type is determined based on which specific bands that are present.

For *HLA-C*06* a SNP typing method developed by Nikamo et al. was used in addition to PCR-SSP [94].

3.3.1 Paper I

3.3.1.1 SNP selection and genotyping

SNPs were selected on the basis of previous publications.

All SNPs were genotyped on a 7900HT Fast Real-Time PCR System Instrument by using allele-specific TaqMan MGB probes labelled with fluorescent dyes FAM and VIC (Applied Biosystems, Stockholm, Sweden), according to the manufacturer's protocols. Allelic discrimination was made with the ABI PRISM 7900HT SDS and the SDS 2.2.1 program (Applied Biosystems). Ten percent of the samples were run as duplicates to check for genotyping errors.

*HLA-C*06:02* was determined using 4 SNPs in the HLA-C region (rs10484554, rs3130457, rs6904246 and rs7745906). [94]

3.3.2 Paper II

3.3.2.1 Sequencing of the *IL22* promoter

The *IL22* promoter was sequenced in 12 psoriasis cases. Sequencing was performed with an ABI 3730 (Applied Biosystem) according to manufacturer's protocols and analyzed with ChromasPro v2.4 (<http://en.bio-soft.net/>).

3.3.2.2 SNP selection and genotyping

SNPs in putative transcription binding sites for AhR-ARNT complex were chosen using bioinformatics tools; RAVEN (<http://www.cisreg.ca/cgi-bin/RAVEN/a>) and TFBIND (tfbind.hgc.jp). All SNPs were typed with TaqMan SNP genotyping assay with custom-designed ABI assays (Applied Biosystems, Stockholm, Sweden).

For detection of the AT insert/deletion (rs35774195 AT/-) in combination with SNP (rs10784699 C/T), a custom designed TaqMan assay was used in which one probe bound in the presence of the AT-insert and the other probe in the absence of the AT-insert. The C allele for the SNP rs10784699 was detected using SNP rs2227477 ($r^2 = 1$).

3.3.2.3 Cell transfection and luciferase assay

The luciferase reporter assay is commonly used as a tool to study gene expression at the transcriptional level. In *paper II* differences in transcriptional activity of the high-risk gene variant vs. low-risk gene variant was measured using a luciferase reporter assay.

Luciferases make up a class of oxidative enzymes found in several species that enable the organisms that express them to emit light. The most famous one of these enzymes is the firefly luciferase. Fireflies are able to emit light via a chemical reaction in which luciferin is converted to oxyluciferin by the luciferase enzyme. Some of the energy released by this reaction is in the form of light.

To perform the reporter assay, you clone the regulatory region of your gene-of-interest X upstream of the luciferase gene in an expression vector, introduce that resulting vector DNA into cells, and let the cells grow for a period of time. You then collect the cells, break them open to release all the proteins (including the luciferase), add luciferin and all the necessary cofactors, and measure the enzymatic activity using a luminometer (an instrument that measures light emission from samples and gives you a quantitative reading). Since your gene-of-interest X is fused to the luciferase reporter gene, the luciferase activity can be directly correlated with the activity of X.

3.3.2.4 Functional immunological assays

Isolation of plasma and mononuclear cells from patients with onset of psoriasis before 10 years of age without systemic treatment was performed and patients were grouped into low-risk and high-risk haplotype. Ex vivo stimulated T cells and supernatants were analysed with flow cytometry.

3.3.3 Paper III

Study III was designed as a cross-sectional study in Stockholm, Sweden. Children were included through consecutive recruitment from new referrals and follow-up visits at the department of dermatology at Karolinska University Hospital and from the psoriasis patient association care units in Stockholm. Inclusion criteria were: a confident clinical diagnosis of psoriasis, age between 0-15 years and onset of disease within the past 12 months. Having only scalp or diaper rash psoriasis was not regarded as sufficient for diagnosis.

A structured, questioner-based, physician-led interview was performed in all cases including present and past medical history and family history. All patients underwent a thorough clinical examination. Nail, scalp and genital regions were inspected in all patients. PASI was assessed at inclusion. A rheumatologist examined all children with joint complaints.

Weight (kg) and height (cm) were measured and BMI was classified according to the International Obesity Taskforce criteria [95]. Blood was drawn for biobanking and for measuring gluten antibodies (transglutaminase antibodies [tTG]) IgA, rheumatoid factor (RF), lipids and glucose.

3.3.4 Paper IV

The samples were separated into two groups, (i) Cases (n = 506) and controls (n = 1001) genotyped in a genome-wide association study (GWAS) using an Illumina 550 platform [96] (data for cases not published) and (ii) Sporadic cases (n = 231) not included in the previous GWAS study.

We used the following genotyping methods: (i) HLA imputation was performed by using SNP genotypes from the Type 1 Diabetes Genetics Consortium (T1DGC; n = 5,225), which has demonstrated a high imputation accuracy for classical HLA alleles [97, 98] and (ii) PCR-SSP Olerup SSPTM HLA-C low resolution kit and HLA-A-B-DR-DQ-SSP combi tray according to the manufacturer's instructions (GenoVision Inc., Stockholm, Sweden).

3.4 STATISTICAL ANALYSIS

Statistical analyses for genetic studies were made using PLINK v1.07 [99] and R statistical software v2.15.0 [100]. SNPs were tested for Hardy-Weinberg equilibrium using χ^2 -test. Allelic frequencies of the SNPs in the case and control groups were compared using logistic regression with gender as covariate. Odds ratios (ORs) and 95% confidence intervals were estimated using the most common allele as the referent and are reported for each minor allele. Significant p-values were corrected for multiple testing using the adjust mode as implemented in PLINK v1.07 in *paper I and II* and in *paper IV* by using the Sidák correction, where p values are adjusted using simple functions of the number of tested hypotheses [101]. Linkage disequilibrium (r^2) was analyzed in HaploView v4.2. [102]

Interaction analyses were calculated in R statistical software v2.15.0 in *paper I*.

Haplotype analysis was analyzed using UNPHASED 3.1.3 program with the mode 'test individual haplotypes' [103] in *paper II* and in the hap function in PLINK v1.07 in *paper IV*. Major alleles were used as reference haplotype.

For analysis of cytokine experiments in *paper II*, the paired and non-paired tests were performed with Wilcoxon and Mann-Whitney, respectively.

In *paper III* descriptive analysis was used to determine mean and SD for normally distributed data and median and inter-quartile range for skewed data. Bivariate associations were examined using Chi-square tests for categorical variables. Multivariable analyses were conducted using linear and logistic regression to examine the associations between outcome variables and clinical and demographic explanatory variables. Associations in the multivariable logistic models were presented as odds ratios (OR) with 95% confidence interval (CI). The significance level was specified at 0.05 for all tests. All statistical analyses were performed using SPSS®Statistics software (version 22, 2013; IBM Corporation, New York, USA).

4 RESULTS AND DISCUSSION

Age dependent genetic and phenotypic differences have been shown in many common, complex diseases like inflammatory bowel disease (IBD), celiac disease, multiple sclerosis (MS), asthma, PsA and diabetes mellitus [104-113]. Several susceptibility loci, not previously reported in adults, were discovered using a large cohort of paediatric patients with IBD [105]. Candidate gene studies in IBD have shown NOD2 carriage, the most robustly associated gene in Crohn's disease, to be higher in early onset (≤ 16 years of age) compared with adult-onset Crohn's disease. Within the group of patients with juvenile PsA two subgroups with different age of onset, clinical phenotype and genetic background has been proposed [111, 114]. Filaggrin mutations have been shown to associate stronger with early onset and severe eczema [115]. In psoriasis, early onset has been associated with a higher degree of family history of psoriasis, carriage of *HLA-C*06:02* allele, more severe disease and guttate phenotype [116]. However early onset in psoriasis has been defined as onset before 40 years of age according to the definition by Henseler and Christophers from 1985 and a more detailed stratification for age of onset has been lacking [117].

Studies performed in several chronic inflammatory diseases, suggest that an early age of onset can affect phenotypic presentation, which in turn may reflect differences in underlying pathogenesis in early vs. late onset of disease. These differences could have an impact on the development of co-morbidities, treatment response and prognosis. Thus exploration of underlying genetics in subpopulations stratified for age on onset could help in understanding disease pathogenesis. The overall aim with this thesis was to explore clinical and genetic differences in psoriasis patients stratified for age of onset. Our hypothesis was that onset of psoriasis in childhood and adolescence differs from adult onset in terms of underlying genetics and clinical characteristics.

Stratification according to age of onset reveals novel patterns

In *paper I* we explored age at onset dependent association with *HLA-C*06:02* and *ERAP1* (rs26653, rs30187, and rs27524) in a case-control study. Cases were stratified according to age of onset into four groups (0-9, 10-20, 21-30, > 40). The age groups were chosen in an attempt to reflect biologically significant transitions. In particular, we aimed to explore the impact of puberty [118]. *HLA-C*06:02* is the candidate allele with the strongest association with psoriasis [48] and has been shown to associate stronger with early onset psoriasis and guttate phenotype. The dogma in the psoriasis field has been that the earlier disease onset the stronger association with *HLA-C*06:02*. A GWAS from 2010 found association with *ERAP1* in psoriasis, this association was confined to patients carrying *HLA-C*06:02* [53]. Association with *ERAP1* is biologically highly interesting since it is an aminopeptidase involved in MHC class I peptide processing in the endoplasmic reticulum and has also been shown to be involved in shedding of pro-inflammatory cytokines [119].

Our data, stratified for age of onset, revealed that association with *ERAP1* was confined to cases with onset between 10 and 20 years ($p = 0.00008$, OR 1.59, 95% CI 1.28-1.98) and no

association was detected in cases with onset below 10 years, reflecting genetic heterogeneity within the childhood psoriasis population. Children with disease onset below the age of ten years had a prevalence of *HLA-C*06:02* similar to that of patients with adult onset (21-40 years) whereas the 10-20 year group showed a much stronger OR for *HLA-C*06:02* (although with overlapping 95% CI between 0-9 and 10-20 year group). Thus our data could not verify the prevailing dogma of the earlier the onset, the stronger the association with *HLA-C*06:02*.

Marker	Age at onset					
	Age 0-9		Age 10-20		Age 21-40	
	P	OR	P	OR	P	OR
HLA-C*06 rs10484554	2.5x10 ⁻²⁸	4.66 (3.48-6.24)	2.9x10 ⁻⁷²	6.65 (5.29-8.35)	4.6x10 ⁻⁴⁶	4.25 (3.44-5.24)
HLA-C*06:02	1.2x10 ⁻³³	5.71 (4.19-7.79)	1.3x10 ⁻⁸⁶	8.46 (6.65-10.77)	8.9x10 ⁻⁵⁸	5.52 (4.40-6.92)
ERAP1 rs26653	0.4	1.24 (0.93-1.65)	0.00008	1.59 (1.28-1.98)	0.05	1.27 (1.05-1.54)
ERAP1 rs30187	0.4	1.20 (0.91-1.57)	0.0001	1.46 (1.18-1.80)	1	1.03 (0.85-1.24)
ERAP1 rs27524	0.4	1.12 (0.86-1.47)	0.02	1.28 (1.04-1.58)	1	0.98 (0.81-1.18)

Table 1. Allelic associations for *HLA-C*06* and *ERAP1* in psoriasis patients stratified for age at disease onset (cases vs. controls).

In contrast to findings in the GWAS study, association with *ERAP1* was not dependent on *HLA-C*06:02* in our material (association with rs26653 in all included cases not carrying *HLA-C*06:02*: $p = 0.001$, OR 1.39, 95% CI 1.14-1.70). We could not confirm the interaction between *HLA-C*06:02* and *ERAP1* in our material, using both additive and multiplicative models. Genetic studies in other MHC associated diseases like ankylosing spondylitis (AS) and Bechet's disease has also shown epistasis (gene interaction) of *ERAP1* and disease specific MHC genes [120, 121]. We cannot explain the discrepancy between our findings and published data on *ERAP1* association being dependent on *HLA-C*06:02* and statistical interaction between the two genes. Different populations, sample size and model for interaction are a few factors that may influence results. Since our data indicated departure from additivity (please see figure 2 in *paper I*), lack of power for interaction calculations is the most likely reason for us not being able to confirm interaction between *HLA-C*06* and *ERAP1* in our material. However, although statistical interaction implies a possible biological interaction it is not a proof for biological interaction and the question of true biological interaction between *ERAP1* and *HLA* remains of paramount interest in autoimmune diseases, but may ultimately be better answered via molecular, rather than statistical, investigation.

Stratification for *HLA-C*06* in the GWAS from Strange et al. in 2010 also revealed association for a SNP near *ZAP70* (rs17695937) in *HLA-C*06* positive individuals. *ZAP70* is a tyrosine kinase that binds to the T-cell receptor-associated chain following the T cell

receptor (TCR) engagement of MHC-peptide complexes and plays a critical role in T-cell signalling. Theoretically an interaction between all three loci (*HLA-C*06*, *ERAP1* and *ZAP70*) would fit very nicely with the hypothesis that psoriasis may in part be caused by dysregulation of HLA-restricted CD8⁺ T cells. However, no interaction models including *ZAP70* were presented in the GWAS study. Adding data on *ZAP70* in our material for calculations on interaction, all patients and stratified for guttate or plaque phenotype, did not reveal any significant findings (data not published).

Genetic association to early onset of psoriasis is linked to functional alterations in IL-22 responses in T cells

In *paper II* we wanted to explore association with *IL22* in our psoriasis cohort, in all patients and in subgroups stratified for age of onset. Interleukin-22 (IL-22) is a member of the IL-10 family cytokines and is produced by lymphocytes including both those of the innate and adaptive immune systems, most notably Th17, Th22 and NK cells. The activation of T-helper 17 cells (Th17) in psoriasis was in focus during the design of the study and underscored by genetic association to components in the Th17 pathway such as interleukin 23 receptor (*IL23R*) [122-124] and compelling efficacy of therapies targeting this pathway. Genetic association with *IL22* had not surfaced in GWAS in the Caucasian population. However, *IL22* association with psoriasis had been reported in the Japanese population [125] and a CNV in the *IL22* gene had been found in the Latvian population. [126] A genetic variation promoting high IL-22 production had recently been reported to associate with protection against tuberculosis in the Chinese population, indicative of an evolutionary advantage of carrying a high IL-22-producing genotype [127]. The protective effect was most pronounced in young patients, which made age stratification in our material even more interesting.

Sequencing of the *IL22* promoter in 12 of our psoriasis cases identified a total of 13 SNPs and one INDEL, all of which were previously reported. The aryl hydrocarbon receptor (AhR) is implicated as a master regulator of IL-22 production and SNPs in putative transcription binding sites for AhR-ARNT complex were chosen using bioinformatics tools (*please see material and methods section*).

Disease onset before puberty is preferentially enriched for genetic variants in the IL-22 promoter region

Genotyping in Caucasian cases (n = 1,069) and controls (n = 1,529) for four variants, covering the linkage disequilibrium structure in the investigated predicted putative binding sites for the AhR-ARNT complex in the *IL22* promoter region was performed. Stratification for age of onset revealed association to all four SNPs only in the group of patients with age at onset < 10 years, strongest association was found for rs2227473 (*p* = 0.02, OR 1.55, CI 95% 1.07-2.26). Case-case analysis comparing age at onset <10 to age at onset 10-40 revealed significant differences between age groups. Association was replicated in a confirmation set (age at onset < 10 years, n = 98 controls n = 1,022) and a meta-analysis in discovery and

confirmation samples. Haplotype analysis, with one minor allele present for all four variants, increased OR to 3.80 ($p = 0.001$, 95% CI 1.62-8.21).

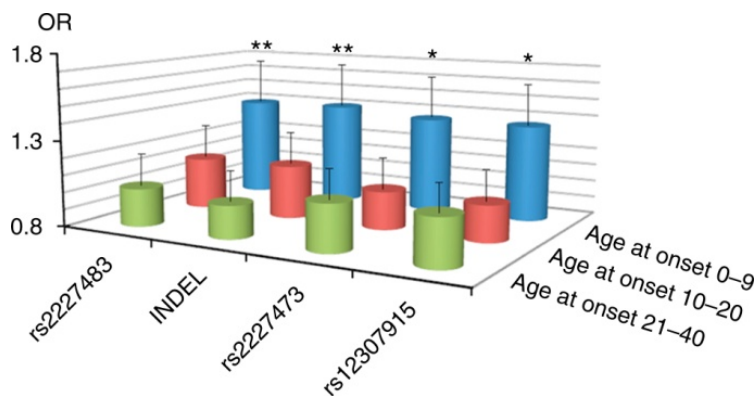


Figure 8. Allelic association to genetic variants in the *IL22* promoter in psoriasis patients stratified for age at disease onset. * $P < 0.05$, ** $P < 0.01$.

Functional consequences of carrying the high-risk haplotype

Functional consequences of carrying the identified high-risk alleles on the expression of *IL22* was shown in a luciferase reporter assay and ex vivo stimulation of CD4 T cells from patients carrying the high-risk haplotype or the low-risk haplotype. Even though the functional experiments do not formally prove the pathogenicity of the proposed high-risk haplotype we detect significant differences between high-risk and low-risk haplotypes. Thus, our data indicate that the genetic background contributed to the immunological signature of circulating T cells.

Thus, stratification for age of onset revealed that disease onset before puberty is preferentially enriched for genetic variants favouring high production of IL-22 and underscores the biological validity of stratification according to age of onset. In the context of disease initiation, early onset psoriasis may be triggered by skin traumas and/or infections in childhood. In high-IL-22-producing individuals, the local levels of IL-22 in the skin may exceed the threshold of keratinocyte activation and create the spark necessary to initiate psoriasis.

However, IL-22, which belongs to the anti-inflammatory cytokine receptor family IL-10, has also been shown to have anti-inflammatory effects in IBD. Inhibition or genetic knockdown of IL-22 worsens the outcomes in models of IBD suggesting a protective role for IL-22 in the gut [128]. Recent data has shown a similar response in psoriasis where activation of the aryl hydrocarbon receptor dampens the severity of inflammatory skin conditions including psoriasis [129]. Thus, the role of IL-22 and the AhR in psoriasis is complex and still incompletely understood.

A cross sectional study of clinical characterization at onset of psoriasis in children.

- Do genetic differences translate into clinical differences?

Our findings in paper I and II that age of onset of childhood psoriasis is inflicted by genetic factors made us interested to study whether this would also translate into phenotypic differences. In particular we wanted to study potential differences between pre-pubertal vs. pubertal onset. That phenotypic characteristics are related to *HLA-C*06* status has previously been shown by Gudjonsson et al. [130] but like most clinical studies, the characterization of childhood psoriasis was retrospective and included patients with long duration of disease. Data captured around the time of onset is lacking.

In **Paper III** we performed a cross-sectional study of clinical characterization in children (< 16 years) with psoriasis at onset (within 12 months of onset). In total 109 patients were eligible for inclusion in the study. Median (min-max) PASI was 5.0 (0.3-18.7). Facial lesions have previously been reported to be more common in children with psoriasis compared to adult patients. [7] In our study 46% of children presented with facial lesions and localisation around the eyes were common. (*Table 2*) Inverse involvement was significantly more common in pre-pubertal children (OR = 2.8, 95% CI = 1.1, 7.1, $p \leq 0.05$) especially in boys (OR = 2.5, 95% CI = 1.1, 6.1, $p \leq 0.05$) independent of phenotype and *HLA-C*06*. *HLA-C*06* associated with the guttate phenotype (OR = 3.4, 95% CI = 1.1, 10.7, $p \leq 0.05$) and facial lesions (OR = 3.8, 95% CI = 1.5, 9.7, $p < 0.01$) and was less common in pre-pubertal ages (OR = 0.28, 95% CI = 0.10, 0.74, $p \leq 0.05$).

During the work of this thesis it has been obvious that finding children within 12 months of onset is not easy. Several patients failed to fulfil the inclusion criteria, onset < 12 months, due to delayed diagnosis.

	All (n = 109)	Pre-pubertal 2-9 years (n = 63)	Pubertal 10-15 years (n = 46)	p-value
Median age, years	8.0	5.8	12.1	
(range)	(2-15)	(2-9)	(10-15)	
Female, n (%)	55 (51)	27 (43)	28 (61)	0.06
Positive family history				
1 st degree relative	46 (42)	25 (40)	21 (46)	0.63
HLA-C*06 positive	71 (65)	35 (56)	36 (78)	0.008
Phenotype				
Plaque	77 (71)	48 (76)	29 (63)	0.052
Guttate	29 (26)	12 (19)	17 (37)	
Pustular	3 (3)	3 (5)	0 (0)	
Facial lesions	50 (46)	33 (52)	17 (37)	0.11
Scalp lesions	96 (88)	55 (87)	41 (89)	0.77
Inverse lesions	45 (41)	35 (56)	10 (22)	<0.001
Nail changes	11 (10)	9 (14)	2 (4)	0.07
Arthritis	4 (4)	3 (5)	1 (2)	
Overweight/Obese	16 (15)	11 (17)	5 (11)	0.32
History of/present				
Flexural eczema	16 (15)	10 (16)	6 (13)	0.68
Allergy	15 (14)	7 (11)	8 (17)	0.35
Asthma	18 (17)	10 (16)	8 (17)	0.83

Table 2. Subject characteristics and bivariate analyses of clinical characteristics by group (pre-pubertal vs. pubertal)

Psoriasis - eczema overlap - filaggrin gene mutations

Several children showed an overlap with “eczema like” lesions and the differential diagnosis for eczema was clearly the most difficult differential diagnosis to distinguish from. In a recent study of childhood psoriasis and eczema, 80% (n = 51) of patients with psoriasis were referred with a tentative diagnosis of atopic dermatitis [131]. Mutations in the filaggrin gene is to date the most significant genetic finding associated with Atopic dermatitis (AD) and exist in 20-40% of moderate-to-severe AD patients with a phenotypic over-representation of early onset of the disease [132]. In collaboration with the group working on AD in our institution we investigated association with filaggrin mutations in our cohort of early onset psoriasis (onset before 16 years). Our data could not reveal any association with filaggrin mutations in children with psoriasis [133].

Pityriasis rubra pilaris and psoriasis – CARD14 gene mutations

Two children initially included were re-evaluated due to atypical presentation and response to treatment and a subsequent diagnosis of juvenile Pityriasis rubra pilaris (PRP) was proposed. Clinical as well as genetic overlap is known for psoriasis and PRP and familial PRP has been

shown to result from gain-of-function mutations in the *CARD14* gene [134, 135]. To test for *CARD14* mutations in sporadic juvenile PRP cases we undertook sequencing of the known *CARD14* mutations in the two patients mentioned above and two additional children from our clinic. No mutations were identified (*data not published*). In 2014 Eytan O et al. published data on *CARD14* mutations in sporadic PRP cases (n = 61). Their data showed increased epidermal expression but absence of mutations in *CARD14* confirming our observations [136].

Differences in association with HLA depending on clinical phenotype and age of onset

In *paper IV* we hypothesized that different HLA alleles could have a stronger effect in some age groups from a biological point of view. We performed a case-control study (cases = 737, controls = 1001) for a more detailed exploration of HLA association (HLA-A, HLA-B, HLA-C, HLA-DR and DQ) in our material stratified for age at onset (0-9 years, 10-20 years, 21-40 years, > 40 years).

Previous studies in autoimmune HLA-associated diseases had shown differences in association with HLA antigens depending on age of onset and clinical phenotype [137-139]. In psoriasis age of onset dependent differences in genetic background had been shown in our data and others [52, 140, 141].

Association with *HLA-A*01* in psoriasis has previously been reported and our data replicated this association. However, stratification for age of onset showed association only in patients with onset of psoriasis before puberty (P = 0.002, OR 1.99 95% CI 1.45-2.75). Haplotype analysis revealed that psoriasis cases more often carried extended haplotype *A*01-C*06* compared to controls and this haplotype was enriched in children with early onset (0-9 years). (*Table 3*)

Table 3: The HLA-A*01 /HLA-C*06 haplotype contributes to enhanced risk for psoriasis onset before 10 years of age

HLA-A*01	HLA-C*06	Age at onset 0-9		Age at onset 10-20	
		P-value	OR	P-value	OR
NN	NN	reference	1	reference	1
P	NN	0.007	0.83 (0.40-1.71)	0.02	0.70 (0.39-1.25)
NN	P	3x10 ⁻⁷	5.42 (3.02-9.74)	5x10 ⁻²⁹	10.44 (6.92-15.75)
P	P	9x10 ⁻¹⁸	10.04 (5.62-17.94)	4x10 ⁻²⁷	12.65 (12.65-19.94)

NN, negative for HLA-A*01 and HLA-C*06, respectively
P, positive for HLA-A*01 and HLA-C*06, respectively
Generalized linear model as implemented in R software package.
Individuals with risk alleles for respective HLA-A*01 and HLA-C*06.

Association with the extended haplotype reported by Schmitt-Egenolf et al. C*06-B*57-DRB1*0701-DQA*0201-DQB1*0303 in patients with onset of psoriasis before 40 years of age was confirmed in our population [142]. However association with the extended haplotype was much stronger in the group of patients with guttate psoriasis in comparison to the group with plaque psoriasis in our material, indicating importance of clinical phenotype for association.

Linkage disequilibrium in the MHC region hampers attempts to find independent associations and adjustment for the strong association with *HLA-C*06* in our material depleted all associations except for *HLA-A*01*, but this association was not significant after adjusting for multiple testing. We conclude that large multicentre studies are needed to further analyse HLA associations in childhood psoriasis and the number of cases is the largest limitation of our study. From a biological point of view it is tempting to speculate that various environmental factors, like infectious panorama of different age periods in life, in combination with specific HLA repertoire may influence age of onset of psoriasis and further studies in this interesting field are warranted.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis the hypothesis that genetic background influences age of onset of psoriasis and disease phenotype was tested. Overall our studies with stratification according to age of onset revealed novel patterns of genetic association to several genes associated with psoriasis.

Differences in genetic background exist between different age of onset groups in psoriasis.

The age cut off at 40 years of age between early and late onset psoriasis proposed by Henseler and Christophers in 1985 seems biologically important. However, within the group of patients with onset < 40 years genetic heterogeneity was found, with differences even within the group of patients with early onset in childhood or adolescence (0-9 vs. 10-20). Further studies in larger international, multi-centre cohorts of early onset psoriasis will be needed to dissect the genetic profile in children and adolescence with psoriasis.

Genetic background influences clinical phenotype. Differences in genetic background could have an impact on the development of co-morbidities, treatment response and prognosis. Future studies following disease development will be important to investigate if genetic differences translate into clinical differences and influences prognosis, development of co-morbidities and treatment response. Thus, exploration of the genetic background in stratified materials may help in determining prognostic genotype, finding biomarkers for disease prognosis and progression and tailored biologic treatment for patients in the future.

To reach statistical power in studies of childhood psoriasis international collaboration and multicentre studies are needed. Since the development of psoriasis depends on both environmental and genetic factors, the individual effect of a single polymorphism is expected to be small. Large and well-characterized populations are therefore crucial for the identification of robust associations. The importance of collaborative multicentre initiatives is obvious in genetic and clinical studies of childhood psoriasis to reach statistical power.

A delay in correct diagnosis for children with psoriasis is common. To establish a confident diagnosis of psoriasis in children can be challenging. Especially differential diagnosis for eczema and PRP can be difficult. Information and education on childhood psoriasis is an important task for us working in the field. Students in medical school at Karolinska Institutet are tutored to refer children with psoriasis to a dermatological unit. I believe this is important in our aim to learn more about disease presentation, progression and treatment response in children and to shorten the delay in correct diagnosis and therapy for these children. Possibly this recommendation should be included in national and international guidelines.

Treatment options for psoriasis are steadily increasing. The hypothesis that early immune modulatory treatment may influence disease development is very interesting and has proven important in other autoimmune diseases. To include children in future drug trials and to follow children on systemic therapies in registries are important to better understand psoriasis in children/adolescence and provide better and safe treatment options.

6 LIMITATIONS

Replication

Replication of findings in association studies is of great importance to validate results. The risk of false positive results in association studies depends on several variables; population stratification, linkage disequilibrium differences in different populations, gene-gene/gene-environment interactions that may differ between populations and lack of power [143, 144]. Because most disease associated alleles in complex diseases have modest or weak effect on disease susceptibility large sample sizes and/or meta-analyses of multiple studies are often required to determine whether genetic associations between polymorphisms and disease are significant.

Association with *HLA-C*06*, *ERAP1*, *IL22* and HLA-alleles in paper IV have previously been reported in other populations of psoriasis patients. However, our findings of age dependent associations in psoriasis has only partially been replicated (*HLA-C*06* and *ERAP1*) from a Dutch group [145]. Thus, additional replication of our findings in larger cohorts is needed for validation.

Functional studies exploring the effect of genetic associations with disease manifestations

An aspect of genetic association studies is the difficult question of when an association is to be considered an association of functional importance. *HLA-C*06:02* is the strongest candidate allele in psoriasis and association with *ERAP1* polymorphisms is theoretically very interesting and has been replicated in several cohorts of psoriasis patients. How *HLA-C*06:02* and *ERAP1* contributes to disease is, however, still unclear. Thus, functional effects of the genetic association findings and differences between age of onset groups remains to be explored.

Sample size and the risk for selection bias in paper III, a cross-sectional study

According to previous studies a large proportion of psoriasis patients develop visible psoriasis lesions before 25 years of age and onset before 10 years has been reported in 10% of patients. Data from health insurance organizations show a linear increase in psoriasis prevalence during childhood and adolescence (0-18 years), with prevalences ranging from 0.1% at the age of 1 year to 0.8% at the age of 18 years [10]. In a population-based study from Minnesota, USA, the overall age- and sex-adjusted annual incidence of paediatric (< 18 years) psoriasis was 40.8 per 100,000 (95% CI: 36.6-45.1). When psoriasis diagnosis was restricted to dermatologist-confirmed subjects in the medical record, the incidence was 33.2 per 100,000 (95% CI: 29.3-37.0) [146].

Based on population data from 2014 from the Swedish administrative agency Statistics Sweden ~ 395 000 individuals aged 2-16 years were posted in Stockholm County. Assuming a psoriasis prevalence of between 0,1-0,8% of the pediatric population <18 years, patients included in our cohort from 2007-2013 only represented a fraction of children with psoriasis

in Stockholm County. This brings risk of selection bias regarding for example disease severity, assuming that the more severely affected cases are likeliest to seek medical help. However, median (min–max) PASI was 5.0 (0.3–18.7) (*paper III*), thus indicating not only children with extensive psoriasis were included. Having a family history of psoriasis may also have influenced the tendency to seek health care. Difficulties in assessing psoriasis diagnosis in infants imply this group is likely underrepresented in the cohort.

In *paper III* the small number of patients, the lack of controls for overweight/obesity and the absence of data on BMI and socioeconomic status in parents, known to be the most predictive factors for overweight/obesity in children, obviously necessitates a cautious interpretation of our results.

Guttate psoriasis

The definition of guttate psoriasis differs between studies in the field of psoriasis research and no distinct definition is used. Also patients can change from one phenotype to another over time. To evaluate type of clinical phenotype, distinguishing between plaque and guttate phenotype is not always a clear cut. For the definition used in the articles in this thesis please see methods section in *paper III*. For the genetic studies in this thesis patients were regarded as guttate phenotype if they had had a guttate episode.

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